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Division of Forensic Science TOXICOLOGY TECHNICAL PROCEDURES MANUAL	Amendment Designator:
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<p style="text-align: center;">45 BENZODIAZEPINE SCREEN AND QUANTITATION BY HPLC-DAD</p> <p>45.1 Summary</p> <p>45.1.1 Benzodiazepines are extracted from biological samples by adding sodium carbonate buffer and extracting with 1-chlorobutane. An aliquot of the extract is analyzed by high performance liquid chromatography-diode array detector (HPLC-DAD).</p> <p>45.2 Specimen Requirements</p> <p>45.2.1 1 mL blood, fluid or tissue homogenate.</p> <p>45.3 Reagents and Standards</p> <p>45.3.1 Diazepam, 1 mg/mL</p> <p>45.3.2 Nordiazepam, 1 mg/mL</p> <p>45.3.3 Clonazepam, 1 mg/mL</p> <p>45.3.4 Alprazolam, 1 mg/mL</p> <p>45.3.5 Lorazepam, 1 mg/mL</p> <p>45.3.6 Temazepam, 1 mg/mL</p> <p>45.3.7 Clobazam, 1 mg/mL</p> <p>45.3.8 Sodium carbonate</p> <p>45.3.9 1-chlorobutane</p> <p>45.3.10 Acetonitrile</p> <p>45.3.11 Potassium phosphate, dibasic</p> <p>45.3.12 Phosphoric acid</p> <p>45.3.13 HPLC grade water</p> <p>45.4 Solutions, Internal Standard, Calibrators and Controls</p> <p>45.4.1 0.2 M Sodium Carbonate: weigh out 10.6 g sodium carbonate, transfer to a 500 mL volumetric flask and QS to volume with deionized water.</p> <p>45.4.2 Mobile Phase A (25 mM phosphate buffer): weigh out 4.35 g of potassium phosphate (dibasic) and transfer to a 1 L volumetric flask filled with approximately 900 mL of HPLC grade water and pH to 5.5 using concentrated phosphoric acid. QS to volume with HPLC grade water. Filter before use.</p> <p>45.4.3 75:25 Phosphate buffer (25 mM):acetonitrile: mix 75 mL 25 mM phosphate buffer with 25 mL acetonitrile.</p> <p>45.4.4 Drug stock solutions:</p>	

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45.4.4.1 If 1 mg/mL commercially prepared stock solutions are not available, prepare 1 mg/mL solutions from powders. Weigh 10 mg of the free drug, transfer to a 10 mL volumetric flask and QS to volume with methanol. Note: If using the salt form, determine the amount of the salt needed to equal 10 mg of the free drug, and weigh this amount. Stock solutions are stored capped in a refrigerator and are stable for 2 years.

45.4.5 The following are examples of acceptable procedures for the preparation of calibrators. Other quantitative dilutions may be acceptable to achieve similar results:

45.4.5.1 Working benzodiazepine standard solution (0.02 mg/mL). Pipet 200 μ L of 1 mg/mL benzodiazepine stock solutions (diazepam, nordiazepam, clonazepam, alprazolam, lorazepam and temazepam) into a 10 mL volumetric flask and QS to volume with methanol.

45.4.5.2 Working internal standard solution (10 mg/L clobazam): Pipet 100 μ L of the 1 mg/mL stock solution of clobazam into a 10 mL volumetric flask and QS to volume with methanol.

45.4.5.3 To prepare the calibration curve, pipet the following volumes of the 0.02 mg/mL working benzodiazepine standard solution into appropriately labeled 16 x 125 mm screw cap test tubes. Evaporate to dryness under nitrogen. Add 1 mL blank blood to obtain the final concentrations listed below.

Amount of stock solution (μ L)	Final concentration of benzodiazepines (mg/L)
100	2.0
50	1.0
20	0.4
10	0.2
5	0.1
2.5	0.05

45.4.6 Controls

45.4.6.1 Benzodiazepine Control. Control may be from an external source or prepared in house using drugs from different manufacturers, lot numbers or prepared by a chemist different than the individual performing the extraction.

45.4.6.2 Negative control. Blood bank blood or equivalent determined not to contain benzodiazepines.

45.5 Apparatus

45.5.1 Test tubes, 16 x 125 mm, round bottom, borosilicate glass with Teflon caps

45.5.2 Test tubes, 16 x 100 mm, round bottom, borosilicate glass

45.5.3 Centrifuge capable of 2000-3000 rpm

45.5.4 Evaporator/concentrator

45.5.5 Vortex mixer

45.5.6 GC autosampler vials with inserts

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45.5.7 pH meter

45.5.8 HPLC-DAD: Agilent Model 1100 HPLC-DAD

45.5.8.1 HPLC Instrument Conditions. The following instrument conditions may be modified to adjust or improve separation and sensitivity.

45.5.8.1.1 Elution Conditions

45.5.8.1.1.1	Column:	Agilent C-18 150 mm x 3 mm, 5 µM particle size	
45.5.8.1.1.2	Column thermostat:	35° C	
45.5.8.1.1.3	Solvent A:	25 mM phosphate buffer (pH 5.5)	
45.5.8.1.1.4	Solvent B:	acetonitrile	
45.5.8.1.1.5	Initial Flow Rate:	0.60 mL/min	
45.5.8.1.1.6	Injection vol.:	35 µL with wash vial	
45.5.8.1.1.7	Stop time:	33 min	
45.5.8.1.1.8	Gradient:	initial	25% B
		15 minutes	32% B
		20 minutes	35% B
		25 minutes	50% B
		27 minutes	50% B
		30 minutes	25% B
45.5.8.1.1.9	Wavelength:	240 nm	

45.6 Procedure

45.6.1 Label clean 16 x 125 mm screw cap tubes appropriately with calibrators, controls and case sample IDs.

45.6.2 Prepare calibrators and controls.

45.6.3 Add 1.0 mL case specimens to the appropriately labeled tubes.

45.6.4 Add 100 µL of the 10 mg/L clobazam internal standard working solution to each tube.

45.6.5 Add 1 mL sodium carbonate and 6 mL 1-chlorobutane to each tube.

45.6.6 Cap and rotate tubes for 30 minutes.

45.6.7 Centrifuge at approx 2800 rpm for 15 minutes. Transfer organic (upper) layer to appropriately labeled tubes.

45.6.8 Evaporate samples to dryness at approximately 50° C under nitrogen.

45.6.9 Reconstitute samples in 100 µL 75:25 phosphate buffer (25 mM):acetonitrile. Transfer to LC autosampler vials for analysis.

45.7 Quality Control and Reporting

45.7.1 This procedure has an LOD of 0.05 mg/L, LLOQ of 0.10 mg/L and ULOQ of 2.0 mg/L.

45.7.2 This procedure can also be used to quantify other benzodiazepines such as midazolam, oxazepam and triazolam using separate calibrator/control solutions. See validation file for relative retention times and spectra.

45.7.3 Benzodiazepines should be confirmed by GC/MS (see Sections 5 and/or 10).

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<p>45.7.4 See Toxicology Quality Guidelines</p> <p>45.8 References</p> <p>45.8.1 IM McIntyre et al. Simultaneous HPLC Gradient Analysis of 15 Benzodiazepines and Selected Metabolites in Postmortem Blood. Journal of Analytical Toxicology. 17: 202-207, 1993.</p> <p>45.8.2 D Flammia, L Edinboro and C Martinez, in house development</p> <p style="text-align: right;">◆End</p>	